



(11) **EP 0 972 566 A2**

(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication:  
**19.01.2000 Bulletin 2000/03**

(51) Int Cl.7: **B01J 20/32**, C07K 1/22,  
B01D 15/08, C08B 37/00

(21) Application number: **99870153.6**

(22) Date of filing: **12.07.1999**

(84) Designated Contracting States:  
**AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU**  
**MC NL PT SE**  
Designated Extension States:  
**AL LT LV MK RO SI**

(72) Inventor: **Tran Quang, Minh**  
**4430 Ans (BE)**

(74) Representative: **Van Malderen, Joelle et al**  
**Office Van Malderen,**  
**Place Reine Fabiola 6/1**  
**1083 Bruxelles (BE)**

(30) Priority: **13.07.1998 US 92654 P**

(71) Applicant: **Tran Quang, Minh**  
**4430 Ans (BE)**

(54) **Affinity immobilised metal resins**

(57) Pentadentate chelators (PDC) resins are the metal chelate resins capable of forming the octahedral complexes with several polyvalent metal ions including  $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Co}^{2+}$  with five coordination sites occupied by the chelator. This results in the best stability of the complexes and in one coordination site free for

interaction and selective binding of accessible cysteine/histidine residues and chiefly histidine containing biomolecules such as proteins or peptides etc.

Cu-PDC can be used as concentration resins to reduce the volume of a protein solution. It can be used also as a universal support for immobilising covalently all proteins, using a soluble carbodiimide.

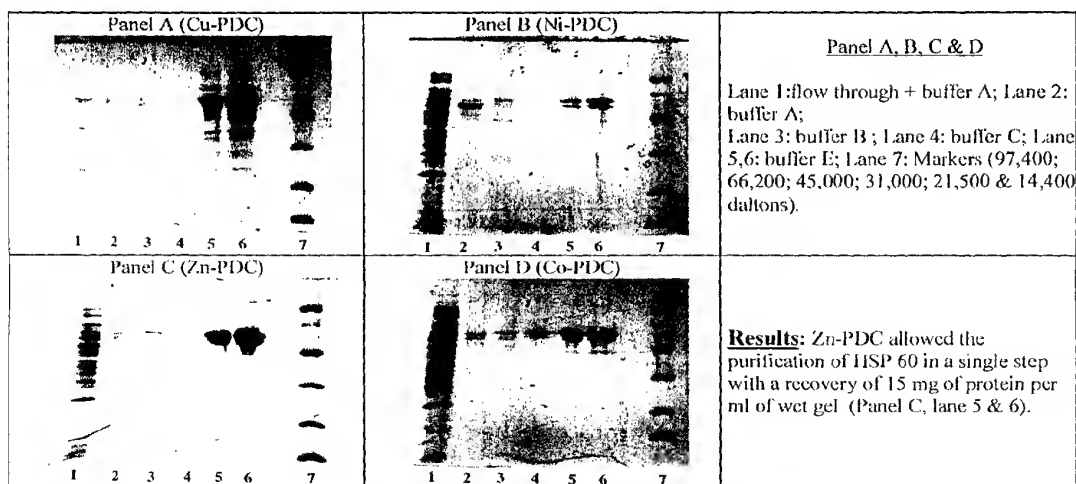


FIG. 1

## Description

### Field of the invention

[0001] The present invention is related to new metal chelator resins and their manufacture process.

### Background of the invention

[0002] Metal Chelate Affinity Chromatography (MCAC) (also denoted Immobilised Metal ion Affinity Chromatography (IMAC)) using affinity immobilised metal resins introduced by Porath et al. (Nature, **258**, 589 (1975) and used for the purification of proteins which contain neighbouring histidine residues, has now become a powerful and versatile tool for the purification of natural and recombinant 6x His-tagged (or not) proteins and peptides.

[0003] The ligand used by these authors was the iminodiacetic acid (IDA). Electron paramagnetic resonance and absorption spectra studies have demonstrated that IDA is a tridentate ligand and the configuration of the complex IDA-M<sup>2+</sup> (1:1) with M<sup>2+</sup>= bivalent metal ions, is a square or tetrahedral one (R. Dallochio et al.; J. Coord. Chem., **25**, 265 (1992). This explains why immobilised IDA can form a stable complex with the ion Cu<sup>2+</sup> and Zn<sup>2+</sup>, but not with other heavy metal ions which need the octahedral configuration for a stable form.

[0004] It is also known that histidine is the only  $\alpha$ -amino acid capable of forming octahedral complexes with different polyvalent metal ions as follows: His - M<sup>2+</sup>- His (B. Rao et al.; J. Inorg. Nucl. Chem.; **33**, 809 (1971); M. M. Harding et al.; Acta Cryst., **16**, 643 (1963):

- Each histidine gives 3 coordination bonds to the M<sup>2+</sup> i.e. the 3-N group of the imidazole ring and the NH<sub>2</sub> and COOH groups of the amino acid; the 1-NH group of the imidazole ring does not participate in the formation of the complexes (C.C. Mc Donald et al.; JACS, **85**, 3736 (1963).
- The complex formation is stereoselective (J.H. Ritma et al.; Recueil, **88**, 411 (1969).
- The complex chemistry of histamine and imidazole has been described (W.R. Walker et al.; J. Coord. Chem., **3**, 77 (1973); Aust. J. Chem., **23**, 1973 (1970).

[0005] Furthermore, Single-crystal X-ray analysis (Simon H. Whitlow; Inorg. Chem., **12**, 2286 (1973)) and Infrared Spectra studies (Y. Tomita et al.; JACS, **36**, 1069 (1963) and J. Phys. Chem., **69**, 404 (1965)) have demonstrated that Trisodium Nitrilotriacetate (Na<sub>3</sub>NTA) is a tetradentate ligand for different polyvalent metal ions M<sup>2+</sup> and the corresponding complexes NTA-M<sup>2+</sup> have an octahedral configuration:

- At pH 5.5-10.0, NTA may be a mixture of HN+(CH<sub>2</sub>-

COO<sup>-</sup>)<sub>3</sub> and N(CH<sub>2</sub>-COO<sup>-</sup>)<sub>3</sub>.

- Only the carboxylate and uncharged N groups participate in the coordination bonding. The carboxylic and charged N groups do not participate in such linkages.

[0006] The NTA derivatives immobilised on Agarose introduced by E. Hochuli et al. (J. Chromatogr., **411**, 177 (1987)) and U.S. patent 4,877,830 (1989) can be therefore, an interesting method for the purification of histidine containing proteins. Their ligands are H<sub>2</sub>N-(CH<sub>2</sub>)<sub>n</sub>-CH(COOH)-N (CH<sub>2</sub>-COOH)<sub>2</sub> (n=2,4) and the resulting resins are: Resin-NH-(CH<sub>2</sub>)<sub>n</sub>-CH(COOH)-N (CH<sub>2</sub>-COOH)<sub>2</sub> (n=2,4).

### Aims of the invention

[0007] The present invention aims to provide new chelator resins having improved characteristics over the compounds of the state of the art and being suitable for metal chelate affinity chromatography.

[0008] The present invention is also related to the preparation process of such resins.

### Summary of the invention

[0009] The present invention is related to an easy, rapid and inexpensive manufacture method of novel resins for IMAC and to said resins being hereafter called Pentadentate chelator (PDC) resins, which advantageously afford 5 coordination bonds to the M<sup>2+</sup> ions. Said coordination bonds may result in a improved stability of the obtained octahedral complexes and one coordination site is free for interaction and selective binding to accessible cysteine/histidine residues and chiefly histidine containing biomolecules that are preferably selected from the group consisting of proteins or peptides.

[0010] Furthermore, said PDC resins are able to chelate with different polyvalent metal ions including Cu<sup>2+</sup>, Ni<sup>2+</sup>, Zn<sup>2+</sup> and Co<sup>2+</sup> to give the corresponding metal chelate resins hereafter called Cu-PDC, Ni-PDC, Zn-PDC and Co-PDC respectively. These four resins will be thereafter used for the purification of histidine containing natural and recombinant proteins or peptides.

[0011] The present invention is also related to said PDC resins, for which the proteins cannot enter into the pores of the resin (molecular weights of proteins are greater than 5000 Daltons, by definition). Preferably, said resin is PDC-Sephadex® G-25 (obtained from Sephadex® G-25, Pharmacia, Uppsala, Sweden).

[0012] In addition, the binding of histidine containing proteins to the chelated metals depends on the complex Metal-PDC resins and the accessibility of histidine residues which in turn, depends on the configuration of the proteins of interest. Therefore, there is no universal rule that will predict the order of magnitude of binding of histidine containing biomolecules to Cu<sup>2+</sup>, Ni<sup>2+</sup>, Zn<sup>2+</sup> and

Co<sup>2+</sup>.

**[0013]** The present invention is also related to a PDC KIT consisting of four separate columns Cu-PDC, Ni-PDC, Zn-PDC and Co-PDC, that determine the most appropriate metal chelate resin suitable for the purification of natural and recombinant biomolecules, preferably selected from the group consisting of proteins or peptides.

**[0014]** The Cu-PDC resins according to the invention are used as universal supports for immobilising covalently proteins using a water-soluble carbodiimide and also as concentration resins to reduce the volume of a protein solution.

**[0015]** A last aspect of the present invention concerns the use of the pentadentate chelator (PDC) resins, and especially the PDC-Sephadex® G-25, according to the invention to obtain water and buffers free of polyvalent metal ions. In particular, the PDC-Sephadex® G-25 according to the invention is useful for preparing "metallo-proteins" free of heavy metal ions or proteins free of heavy metal ions after the Immobilised Metal Ion Affinity Chromatography steps.

#### Detailed description of the invention

**[0016]** As mentioned by Y. Tomita et al. (JACS, 36, p. 1069 (1963) and J. Phys. Chem. **690**, 404 (1965)), at physiological pH, the NTA (nitrilotriacetate) and eventually immobilised NTA derivatives are the mixture of tridentate and tetradentate ligands for the metal ions M<sup>2+</sup>. The concentration of octahedral complexes NTA-M<sup>2+</sup> may be therefore very smaller than the total NTA one, at physiological pH.

**[0017]** The pentadentate chelator (PDC) resins according to the invention, especially of the following formula, are the ideal solution of this problem: Resin-N(CH<sub>2</sub>-COOH)-(CH<sub>2</sub>)<sub>4</sub>-CH(COOH)-N(CH<sub>2</sub>-COOH)<sub>2</sub>. At physiological pH, said resins are a mixture of tetradentate and pentadentate ligands for the metal ions M<sup>2+</sup>. The concentration of octahedral complexes PDC-M<sup>2+</sup> is therefore optimal as well as their corresponding capacity for histidine containing proteins.

**[0018]** The present invention is also related to the manufacture process of a compound of formula: Resin-ω-N-Lysine synthesised by reaction between Bis-Lysine-M<sup>2+</sup>, especially the Bis-Lysine-Cu<sup>2+</sup> and an activated resin of formula: Resin-O-CH<sub>2</sub>-ethylenepoxide or any other activated matrix being able to react with -NH<sub>2</sub> containing organic compounds. Preferably, the carrier matrix used in the above process can be any functionalised or activated resins used for the manufacture of affinity resins, preferably a resin selected from the group consisting of Sepharose® CL-4B, CL-6B, Fast Flow, and Sephadex® G-25 resins (Pharmacia, Uppsala, Sweden), Cellulose and/or Cotton.

**[0019]** The present invention concerns also a reaction process of said Resin-ω-N-Lysine with an excess of halogenoacetic acid, preferably bromoacetic acid, in a basic medium, which allows the formation of the pentaden-

tate resin according to the invention: Resin-ω-N-Lysine + Br-CH<sub>2</sub>-COOH in basic media → Resin-N(CH<sub>2</sub>-COOH)-(CH<sub>2</sub>)<sub>4</sub>-CH(COOH)-N(CH<sub>2</sub>-COOH)<sub>2</sub>.

**[0020]** The present invention will be described in details in reference to the enclosed non-limiting examples.

#### Examples

##### Example 1

**[0021]** 75 g of Lysine monochlorhydrate were dissolved in a solution of 33 g of sodium hydroxide and 330 ml of distilled water. To this solution, was added a solution of 51.6 g of CuSO<sub>4</sub> in 150 ml of distilled water (heated at 30°C until complete dissolution). The corresponding complex was used for the following operations without purification. (However, purification could be carried out by adding ethanol until the formation of a non-miscible phase).

##### Example 2

**[0022]** 300 ml of Sepharose® CL-4B abundantly washed with distilled water, were activated with 195 ml of NaOH 2M diluted in 450 ml of distilled water and 75 ml of epichlorhydrin at 40°C for 2 hours. The corresponding activated resin was washed with distilled water until neutral pH was achieved. To this activated resin, were added 150 ml of NaOH 2M and the solution prepared in Example 1. The mixture was stirred mildly at 40°C for 3 hours and then at 50°C overnight.

**[0023]** The resulting resin was washed abundantly with the distilled water until the pH of the waste water reached 7.0, then abundantly with an aqueous diluted acid solution and finally with an excess of distilled water, until the complete decoloration of the resin. This resin was sufficiently pure for the following operations.

##### Example 3

**[0024]** To 300 ml of the resin prepared in Example 2, were added 405 ml of NaOH 2M and a solution of 75 g of bromoacetic acid and 270 ml of NaOH 2M. The mixture was stirred at 4°C for 3 hours and then overnight at room temperature. The resin was washed abundantly with distilled water until the pH of the wastewater reached 7.0 and was stored in NaCl 0.5M, in the presence of NaN<sub>3</sub> 0.02% (w/v).

##### Example 4

**[0025]** To a solution of 10 g of MCl<sub>2</sub> or MSO<sub>4</sub> (M = Cu or Zn or Ni or Co) in 800 ml of distilled water, were added 300 ml of the resin prepared in Example 3. The mixture was stirred gently for 5 minutes. The metal chelated resin was filtered off, washed 3 times with 500 ml of distilled water, 3 times with 500 ml of NaH<sub>2</sub>PO<sub>4</sub> 0.1M pH 4.0, 3 times with 500 ml of NaH<sub>2</sub>PO<sub>4</sub> 0.1M pH 8.0 and finally

once with 500 ml of  $\text{NaH}_2\text{PO}_4$  0.1M pH 7.5. The resin was stored in  $\text{NaH}_2\text{PO}_4$  0.1M pH 7.5 in the presence of  $\text{NaN}_3$  0.05% (w/v). The such obtained resins were named respectively Cu-PDC, Ni-PDC, Zn-PDC and Co-PDC.

#### **Example 5**

[0026] Four resins, i.e. Cu-PDC, Ni-PDC, Zn-PDC and Co-PDC obtained in Example 4, were loaded separately into four small polyethylene columns to reach 1 ml of resin in each. The set of these four columns was named the PDC KIT. In the following examples 6, 7, 8, 9, 10, the purification of the proteins of interest using the PDC KIT was obtained as follows.

- Each column was equilibrated with 2 ml of the phosphate buffered saline (PBS)  $\text{NaH}_2\text{PO}_4$  50mM, NaCl 300mM pH 7.5;  $\text{NaN}_3$  0.1% (w/v) (buffer A).
- The crude clarified lysate containing the protein of interest in buffer A, was loaded onto each column as indicated in each case.
- Each column was then washed three times with 2 ml of buffer A, three times with 2 ml of buffer B (buffer A + urea 4M pH 7.5), three times with 2 ml of buffer C (buffer A + urea 8M pH 7.5), once with 2 ml of buffer D (buffer A adjusted to pH 6.0) and finally once with 2 ml of buffer A.
- Each column was eluted three times with 2 ml of buffer E (buffer A + imidazole 100mM pH 7.5) and three times with 2 ml of buffer F (buffer A + imidazole 200 mM pH 7.5).
- The fractions obtained from each step of the purification were assayed using the most appropriate system e.g. O.D. at 280nm, Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), etc.

#### **Example 6: Purification of proteins by using the PDC kit (as described in Example 5)**

[0027]

- The crude clarified lysate of 6x His-tagged HSP 60 (heat shock protein) from *Helicobacter pylori* expressed in *E. coli* (concentration of HSP60: approx. 5 mg/ml)
- Sample volume that was loaded onto each column of PDC KIT: 500 microliter (see Figs.).

#### **Example 7: Purification of proteins by using the PDC kit (as described in Example 5)**

[0028]

- The crude clarified lysate of 6x His-tagged Urease from *Helicobacter pylori* expressed in *E. coli* (concentration of urease: approx. 1 mg/ml)

- Sample volume that was loaded onto each column of the PDC KIT: 2 ml.

#### **Results:**

[0029] Ni - PDC can be used to purify the native urease and Zn - PDC to obtain the  $\alpha$ -chain (MW 60,000 Dalton) and the  $\beta$ -chain (MW 30,000 Dalton) of urease, clearly demonstrated by SDS-PAGE.

#### **Example 8: Purification of proteins by using the PDC kit (as described in Example 5)**

[0030]

- The crude clarified lysate of 6x His-tagged Penicillin binding protein 5 (MW= 70,000 Dalton) from *E. coli* (concentration of Penicillin binding protein 5: approx. 0.1mg/ml)
- Sample volume that was loaded onto each column of the PDC KIT: 2 ml.

#### **Results:**

[0031] Ni - PDC is the best for this purification, clearly demonstrated by SDS-PAGE.

#### **Example 9: Purification of proteins by using the PDC kit (as described in Example 5)**

[0032]

- The crude extract (redissolved ammonium sulfate precipitate) containing a mesophilic alkaline protease MW 50,000 Dalton (Zinc protein) from *Pseudomonas aeruginosa* IFO (Institute of fermentation of Osaka) 3455 (concentration of alkaline protease: approx. 1mg/ml)
- Sample volume that was loaded onto each column of the PDC KIT: 1 ml (see Figs.).

#### **Example 10: Purification of proteins by using the PDC kit (as described in Example 5)**

[0033] Sample volume that was loaded onto the column: 10 ml of crude clarified lysate of mutated triosephosphate isomerase from *E. coli*, containing 8 histidine residues (5 accessible).

#### **Results:**

[0034] Ni-PDC allowed the purification of mutated triosephosphate isomerase in a single step with a recovery of 15 mg of protein / ml of wet gel, clearly demonstrated by SDS-PAGE.

**Example 11**

[0035] A solution of 10 mg of human Thyroxine binding globulin (TBG) dissolved in 50 ml of buffer A (see Example 5), was loaded onto the 1 ml Cu-PDC column. The optical density at 280nm of the flow-through indicated that the totality of TBG was retained by the column. The recovery of TBG eluted by 2 ml of buffer E (see Example 5) was approximately 95% (9.5 mg). Its activity determined by RIA (radioimmunoassay), was revealed unaffected.

**Example 12**

[0036] 20 mg of bovine serum albumin (BSA) dissolved in 5 ml of buffer A (see Example 5), were mixed during 15 minutes with 1 ml of Cu-PDC resin pre-equilibrated with the same buffer. The suspension was filtered off, washed respectively with 5 ml of buffer A adjusted to pH 8.0, 5 ml of buffer A adjusted to pH 4.0 and with 5 ml of buffer A adjusted to pH 5.5.

[0037] The optical density at 280 nm of the filtrates of each washing step indicated that the quasi-totality of BSA was retained by the Cu-PDC resin.

[0038] A solution of 10 mg of 1-ethyl 3-(3-dimethylaminopropyl)carbodiimide hydrochloride dissolved in 1 ml of distilled water, was added to the suspension of 1 ml of complex BSA-Cu-PDC resin obtained previously, in 4 ml of buffer A pH 5.5. The mixture was shaken mildly overnight at 4°C.

[0039] The resin was filtered off, washed abundantly with the buffer A. The  $\text{Cu}^{2+}$  ions were stripped from the resin with EDTA (ethylenediaminetetraacetic acid) 0.1M pH 7.4. The resin was then washed with 25 ml of buffer A adjusted to pH 4.0, 25 ml of buffer A adjusted to pH 8.0 and stored in buffer A pH 7.5.

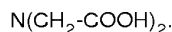
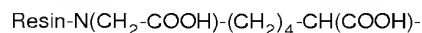
[0040] The recovery of a such covalent immobilisation i.e. BSA-PDC, was quantitative.

**Example 13**

[0041] 100 ml of Ig of bovine serum albumin (BSA) in buffer A (see Example 5) containing 5 mg of  $\text{CuCl}_2$ , 5 mg of  $\text{Ni}_2\text{SO}_4$ , 5 mg of  $\text{ZnCl}_2$ , 5 mg of  $\text{CoCl}_2$  and 1 mg of  $\text{CaCl}_2$  was loaded onto a large section column containing 10 ml of PDC-Sephadex<sup>R</sup> G-25. The solution of BSA such obtained was free of polyvalent metal ions.

**Claims**

1. A pentadentate chelator resin (PDC resin) of formula:



2. PDC resin according to claim 1, having a resin with

pores which do not allow penetration by proteins having a molecular weight higher than 5000 Daltons.

- 5 3. A metal pentadentate chelator ( $\text{M}^{2+}$  PDC) resin according to claim 1 or 2, wherein  $\text{M}^{2+}$  is a polyvalent metal ion selected from the group consisting of  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ni}^{2+}$  and  $\text{Co}^{2+}$ .
- 10 4. A pentadentate chelator kit (PDC-kit) consisting of four separated metal pentadentate chelator ( $\text{M}^{2+}$  PDC) resins wherein  $\text{M}^{2+}$  is respectively the polyvalent metal ion  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ni}^{2+}$  and  $\text{Co}^{2+}$ .
- 15 5. A method for the manufacture of a pentadentate chelator (PDC) resin according to claim 1 or 2, comprising the step of reacting a carrier matrix of a resin with a metal chelator of formula:  
Bis-Lysine- $\text{Cu}^{2+}$ , in order to obtain a compound of formula: Resin- $\omega$ -N-Lysine, being possibly reacted thereafter with an excess of halogenoacetic acid in basic media, and the step of recovering the obtained pentadentate chelator (PDC) resin.
- 20 6. Method according to claim 5, wherein the halogenoacetic acid is the bromoacetic acid.
- 25 7. Intermediate product of formula: Resin- $\omega$ -N-Lysine.
- 30 8. Use of the metal pentadentate chelator ( $\text{M}^{2+}$  PDC) resin according to claim 3 or the PDC kit according to claim 4 for the purification of natural and/or recombinant proteins or peptides, preferably containing histidine residues.
- 35 9. Use of the pentadentate chelator (PDC) resin according to claim 2 for the purification of water of buffers from heavy metals ions.
- 40 10. Use of the pentadentate chelator resin according to claim 2 for the preparation of "metallo-proteins" or proteins free of heavy metals ions after an immobilised metal ions affinity chromatographic step.
- 45 11. Use of the metal pentadentate chelator ( $\text{M}^{2+}$  PDC) resin according to claim 3, wherein  $\text{M}^{2+}$  is the polyvalent metal ion  $\text{Cu}^{2+}$  for a covalent immobilisation of a protein by using a water soluble carbodiimide and for reducing the volume of a protein solution.

50

55

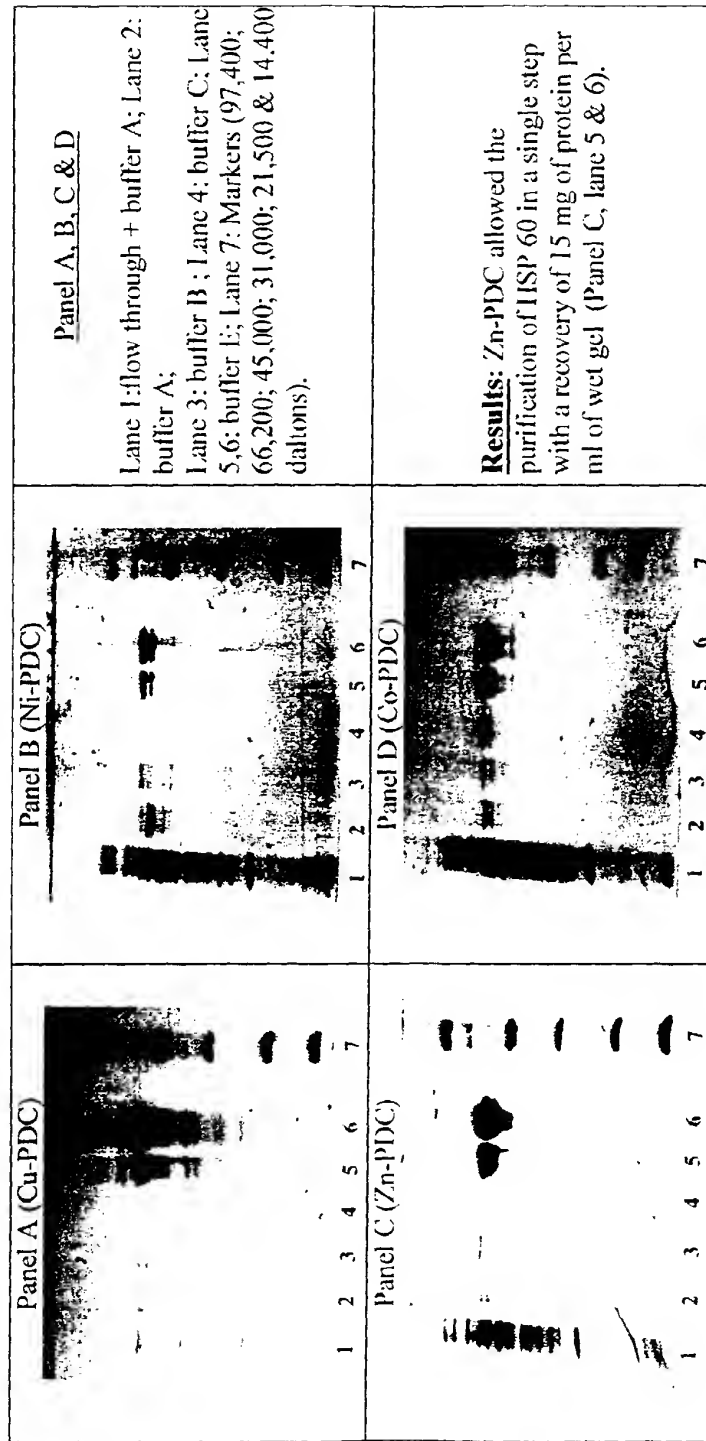


FIG. 1

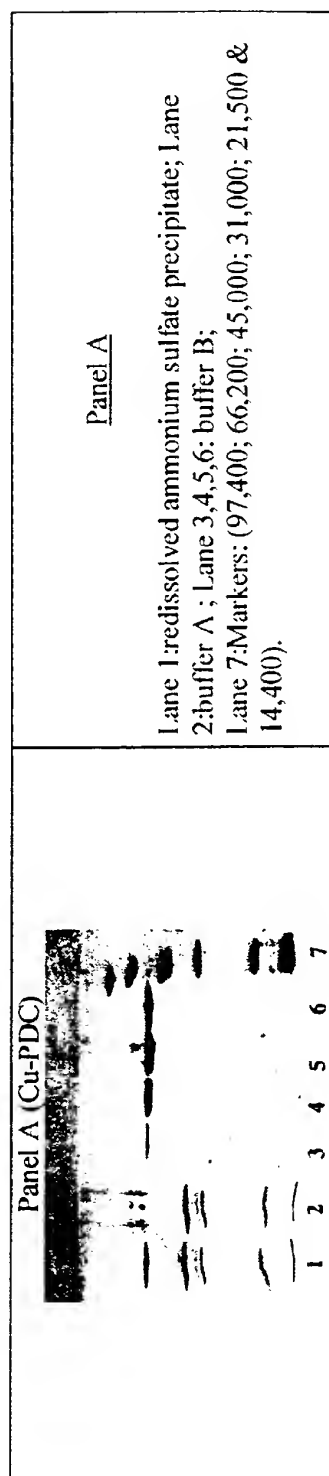


FIG. 2